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(54) **TREATING ALLERGY WITH DETOXIFIED E. COLI HEAT-LABILE ENTEROTOXIN**

BEHANDLUNG VON ALLERGIEN MIT DETOXIFIZIERTEM WÄRMELABILEM E.
COLI-ENTEROTOXIN

TRAITEMENT DE L'ALLERGIE UTILISANT UNE ENTÉROTOXINE THERMOLABILE D'E. COLI
DÉTOXIFIÉE

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(73) Proprietor: **Development Center for
Biotechnology
New Taipei City 221 (TW)**

(72) Inventors:
• **HSU, Yu-Shen
New Taipei City 221 (TW)**
• **WANG, Jiu-Yao
Tainan 70101 (TW)**

(74) Representative: **Kremer, Simon Mark et al
Mewburn Ellis LLP
City Tower
40 Basinghall Street
London EC2V 5DE (GB)**

(56) References cited:
JP-A- 2003 012 556 US-A1- 2003 113 338
US-A1- 2008 102 078 US-B1- 7 208 155

- **HOLMGREN J ET AL: "MUCOSAL ADJUVANTS
AND ANTI-INFECTION AND
ANTI-IMMUNOPATHOLOGY VACCINES BASED
ON CHOLERA TOXIN, CHOLERA TOXIN B
SUBUNIT AND CPG DNA", EXPERT REVIEW OF
VACCINES, FUTURE DRUGS, LONDON, GB, vol.
2, no. 2, 1 April 2003 (2003-04-01), pages 205-217,
XP009060650, ISSN: 1476-0584, DOI:
10.1586/14760584.2.2.205**
- **PIZZA, M. ET AL.: 'Mucosal vaccines; non toxic
derivatives of LT and CT as mucosal adjuvants'
VACCINE vol. 19, 2001, pages 2534 - 2541,
XP027349972**

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Description

BACKGROUND OF THE INVENTION

[0001] Allergic asthma, mediated by pathologic Th2-type immune responses, is a major healthy problem in western countries. Symptoms of allergic asthma include increased mucus production and airway hyper-responsiveness. Repetitive exposure to allergens is an important contributing factor to this disease.

[0002] Steroids and other anti-inflammatory drugs are commonly used for treating allergic asthma. These drugs only alleviate asthma syndromes and do not prevent occurrence of allergic immune responses. Recently, allergy vaccine was developed as a preventive approach. A typical allergy vaccine contains an allergen (e.g., a pollen allergen or a microbial allergen) to induce specific immune responses and an adjuvant (e.g., CpG oligonucleotide or a mutated *E. coli* heat-labile enterotoxin) to enhance the allergen-specific immune responses. For example, JP 2003-012556 A teaches a pharmaceutical composition for treating allergic diseases. The composition comprises an antigen as cause of allergy and an immunoregulating substance (e.g., detoxified cholera toxin (CT)).

SUMMARY OF THE INVENTION

[0003] The present invention is based on an unexpected discovery that a detoxified *E. coli* heat-labile enterotoxin (LT) mutant, either alone or in combination with an allergen, is effective in treating allergic asthma.

[0004] Accordingly, the present invention provides a detoxified *E. coli* heat-labile enterotoxin (LT) for use in the treatment of allergy in a subject via administration of an amount effective for inducing an anti-allergic response in the subject. The detoxified LT of the present invention contains a mutated subunit A that contains an amino acid mutation at the position corresponding to position 53, 61, 63, 69, 97, 104, 106, 110, or 112 in SEQ ID NO:1

[0005] Described herein is a method for treating allergy (e.g., allergic asthma) by administering to a subject in need thereof (e.g., a human suffering from or at risk for allergy) a detoxified LT mutant in an amount effective for inducing an anti-allergic response. The detoxified LT mutant exhibits substantially reduced toxicity as compared to its wild-type counterpart. Namely, its toxicity level does not exceed 1% of that of its wild-type LT counterpart when determined in the conventional Y-1 adrenal cell assay or does not exceed 10% of that of the wild-type counterpart when determined in the conventional Caco-2 cell assay.

[0006] The detoxified LT used in the method described herein can contain a mutated subunit A, e.g., a subunit A mutant including an amino acid substitution at the position corresponding to position 61 or position 63 in SEQ ID NO:1 shown below. LTS61K and LTS63K are two examples. The detoxified LT, alone or combined with an allergen, can be formulated in a pharmaceutical composition to be administered mucosally (e.g., intranasally or sublingually) or by other routes (e.g., transcutaneous delivery).

[0007] Also described is the pharmaceutical composition mentioned above for use in treating allergy by, e.g., mucosal or transcutaneous administration, or for use in manufacturing a medicament for the treatment.

[0008] The details of one or more embodiments of the invention are set forth in the description below. Other features or advantages of the present invention will be apparent from the following drawings and detailed description of several embodiments, and also from the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] The drawings are first described.

Fig. 1 is a diagram showing a preventive protocol and a therapeutic protocol for treating asthma mice with LT mutant LTS61K, allergen Der p, or a combination thereof.

Fig. 2 is a chart showing levels of AHR (represented by Penh values) in mice treated with LTS61K, Der p, or a combination thereof (LTS61K/Der p) following the preventive protocol shown in Fig. 1. *: $p < 0.05$ (LTS61 K-Der p versus normal saline). +: $p < 0.05$ (LTS61K/Der p versus Der p alone). ++: $P < 0.01$ (LTS61 K/Der p versus Der p alone). The p values were obtained by two-way ANOVA. LT represents LTS61K.

Fig. 3 is a diagram showing the levels of certain cytokines (A: IL-5; B: TNF- α ; C: IL-12; D: Eotaxin; and E: TARC) in Bronchoalveolar lavage (BAL) fluids of the mice treated with LTS61K, Der p, or a combination thereof (LTS61 K/Der p) following the therapeutic protocol shown in Fig. 1. *: $p < 0.05$ (LTS61K versus control or LTS61K/Der p versus normal saline). +: $p < 0.05$ (LTS61K versus Der p alone or LTS61K/Der p versus Der p alone). ++: $p < 0.01$ (LTS61K versus Der p alone or LTS61K/Der p versus Der p alone). The p values were obtained by Student t-test. LT represents LTS61K.

Fig. 4 is a diagram showing serum (panel a) and BAL fluid (panel b) levels of Der p-specific IgA in the mice treated with LTS61K, Der p, or a combination thereof (LTS61K/Der p) following the preventive protocol shown in Fig. 1. *:

$p < 0.05$ (LTS61K/Der p versus Der p alone). ++ : $p < 0.01$. (LTS61K/Der versus normal saline). The p values were obtained by Student t-test. LT represents LTS61K.

Fig. 5 is a diagram showing serum (panel a) and BAL fluid (panel b) levels of Der p-specific IgA in the mice treated with LTS61K, Der p, or a combination thereof (LTS61K/Der p) following the therapeutic protocol shown in Fig. 1. *: $p < 0.05$ (LTS61K/Der p versus Der p alone). +: $p < 0.05$ (LTS61K/Der p versus normal saline). The p values were obtained by Student t-test. LT represents LTS61K.

DETAILED DESCRIPTION OF THE INVENTION

[0010] Described herein is a method for treating allergy (e.g., allergic asthma) with an effective amount of a detoxified LT mutant, either taken alone or in combination with an allergen. The term "treating" as used herein refers to the application or administration of a composition including one or more active agents to a subject, who has an allergic disease, a symptom of the allergic disease, or a predisposition toward the allergic disease, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the disease, the symptoms of the disease, or the predisposition toward the disease. "An effective amount" of the detoxified LT mutant used in the methods described refers to the amount of the LT mutant required to confer therapeutic effects on a subject, i.e., inducing an anti-allergic response, thereby alleviating allergic symptoms in the subject. Anti-allergic responses include inhibiting infiltration and recruitment of activated immune cells associated with allergic responses, enhancing secretion of IgA (particularly non-antigen specific IgA), reducing TARC secretion, and suppressing Th2-type immune responses. Th2-type immune responses are characterized by the release of Th2-type cytokines (e.g., IL-4, IL-5, IL-10, and IL-13), leading to generation of humoral immunity, activation of eosinophil, regulation of cell-mediated immune response, and stimulation of IgE production. Effective amounts vary, as recognized by those skilled in the art, depending on route of administration, excipient usage, and co-usage with other active agents.

[0011] A detoxified LT mutant suitable for use in the methods described exhibits substantially reduced cytotoxicity relative to its wild-type counterpart, as determined in either the Y-1 adrenal cell assay or the Caco-2 cell assay, both of which are well known in the art. See, e.g., David et al., 1975, Infection and Immunity, 11:334-336; Cheng et al., 1999, Vaccine 18:38-49; Grant, C.C. et al., 1994, Infection and immunity, 62:4270-4278; Cheng, E. et al, 2000, Vaccine, 18:38-49; and Park, E. J. et al, 1999, Experimental and molecular medicine, 31: 101-107. Below are brief descriptions of these two assays.

[0012] To perform the Y-1 adrenal cell assay, mouse Y-1 adrenal tumor cells (ATCC CCL-79), preferably maintained in Ham's F12 media supplemented with 1.5% horse serum, 2.5% fetal bovine serum, 2mM L-glutamine, and 1.5 g/L sodium bicarbonate, can be seeded in 96-well flat-bottom plates at a concentration of 2×10^4 cells per well (200ul/well) at 37°C in 5% CO₂ for 48 hrs. The cells are washed several times with 1x PBS (pH 7.4) and then treated with a test LT (either wild-type LT or mutated LT) at various concentrations under 37°C with 5% CO₂ supply. Around 24 hours after the treatment, the cells can be observed under a light microscope to examine occurrence of cell rounding. The toxicity of the LT in the test sample is defined as the minimum LT concentration required to initiate cell rounding (EC_i) or the LT concentration required for inducing 50% cell rounding (EC₅₀).

[0013] The Caco-2 cell assay can be performed as follows. Caco-2 cells (ATCC HTB-37) are maintained in MEM- α medium supplemented with 20% FBS in a 24-well plate at a concentration of 5×10^4 cells per well. When the cells are close to 100% confluency, the culture medium is replaced with MEM- α supplemented with 1% FBS and 1 mM 3-isobutyl-1-methylxanthine (IBMX). After being incubated for 30 min with 5% CO₂ supply, the cells are mixed with a test LT and incubated for 4 hours. The cells are washed several times with cold PBS, mixed with 200 μ l of 0.1 N HCl for 15 minutes at room temperature, and then lysed, the supernatants collected. The levels of the cAMP in the supernatants, representing the toxicity level of the test LT, are determined by ELISA using, e.g., the kit provided by Assay designs; Correlate-EIA.

[0014] The detoxified LT mutant can be prepared by introducing one or more mutations into a wild-type LT, either in its subunit A or in its subunit B, via conventional genetic engineering technology. Shown below are the amino acid sequences of a wild-type subunit A (mature form) and a wild-type subunit B, and a nucleotide sequence encoding both the A and B subunits.

Amino acid sequence (SEQ ID NO:1) of mature LT_A

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NGDKLYRADS RPPDEIKRSG GLMPRGHNEY FDRGTQMNIN LYDHARGTQT GFVRYDDGYV 60
STSLSLRSAH LAGQSILSGY STYYIYVIAT APNMFNVNDV LGVYSPHPYE QEVSALEGGIP 120
YSQIYGWYRV NFGVIDERLH RNREYDRYY RNLNIAPAE DGYRLAGFPPD HQAWREEPWI 180
HHAPQCGGNS SRTITGDTCT EETQNLSTIY LRKYQSKVKR QIFSDYQSEV DIYNRI RNEL 240

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Amino acid sequence (SEQ ID NO:2) of LT_B

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MNKVKCYVLF TALLSSLCAY GAPOSITELC SEYRNTQIYT INDKILSYTE SMAGKREMI 60
ITFKSGATFQ VEVPGSQHID SQKKAIERMK DTLRITYLTE TKIDKLCVWN NKTPNSIAAI 120
SMEN

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Nucleotide sequence (SEQ ID NO:3) of LT (Subunit A: 1-777; Subunit B: 774-1148)

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atgaaaaata taacttttcat ttttttttatt ttatttagcat cgccattata tgcaaatggc 60
gacaaaattat accgtgctga ctctagaccc ccagatgaaa taaaacgttc cggaggtctt 120
atgcccagag ggcataatga gtacttcgat agaggaactc aaatgaatat taatctttat 180
gatcacgcga gaggaacaca aaccggcttt gtcagatatg atgacggata tgtttccact 240
tctcttagtt tgagaagtgc tcacttagca ggacagtcta tattatcagg atattccact 300
tactatatat atgttatagc gacagcacca aatatgttta atgttaatga tgtattaggc 360
gtatacagcc ctcacccata tgaacaggag gtttctgcgt taggtggaat accatattct 420
cagatatatg gatggtatcg tgttaatttt ggtgtgattg atgaacgatt acatcgtaac 480
agggaatata gagaccggta ttacagaaat ctgaatatag ctccggcgaga ggatggttac 540
agattagcag gtttcccacc ggatcaccaa gcttggagag aagaaccctg gattcatcat 600
gcaccacaag gttgtggaaa ttcatacaaga acaattacag gtgatacttg taatgaggag 660
accagaatc tgagcacaat atatctcagg aaatatcaat caaaagttaa gaggcagata 720
ttttcagact atcagtcaga ggttgacata tataacagaa ttcggaatga attatgaata 780
aagtaaaatg ttatgtttta tttacggcgt tactatcttc tctatgtgca tacggagctc 840
cccagtttat tacagaacta tgttcggaat atcgcaacac acaaatatat acgataaatg 900
acaagatact atcatatacg gaatcgatgg caggcaaaaag agaaatggtt atcattacat 960
ttaagagcgg cgcaacattt caggtcgaag tcccgggcag tcaacatata gactcccaaa 1020
aaaaagcoat tgaaaggatg aaggacacat taagaatcac atatctgacc gagacccaaa 1080
ttgataaatt atgtgtatgg aataataaaa cccccaattc aattgcggca atcagtatgg 1140
aaaactag

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[0015] The detoxified LT mutant contains a mutated A subunit that includes a mutated residue at a position essential to its ADP-ribosyltransferase activity, e.g., position 53, 61, 63, 69, 97, 104, 106, 110, or 112 in SEQ ID NO:1. See US Patent 6,149,919, US 20081012078, Cieplak et al., J. Biol. Chem. 270(51):30545-30550, 1995; and Cheng et al., Vaccine 18:38-49, 2000. In the LT mutant, the A and B subunits form the typical ABs holotoxin structure. In one example, the detoxified LT mutant (LTS61K) contains a mutated subunit A that includes K at position 61 in SEQ ID NO:1 and a wild-type subunit B. In another example, the detoxified LT mutant (LTS63K) contains a mutated subunit A that includes K at position 63 in SEQ ID NO:1 and a wild-type subunit B.

[0016] Other detoxified LT mutants are disclosed in US2001/0044416, Nawar et al., Infection and Immunity 73(3): 1330-1342 (2005), Komase et al., Vaccine 16(2):248-254, 1993; Magagnoli et al., Infection and Immunity 64(12):5434-5438, 1996; WO99/47164, and US Patent 6,818,222.

[0017] A detoxified LT mutant can be prepared via conventional recombinant technology. For example, nucleotide sequences coding for both the A and B subunits of an LT mutant can be inserted into an expression cassette and expressed in a host cell (e.g., an *E. coli* cell). The A and B subunits can be self-resembled in the host cell to form a holotoxin, which can be isolated from the host cell via, e.g., chromatography. See US20081012078. The toxicity of the LT mutant thus prepared can be determined in either the Y-1 adrenal cell assay or the Caco-2 cell assay, both described above and those displaying substantially reduced toxicity are identified for use in the method described herein.

[0018] Any of the detoxified LT mutants, either alone or in combination with one or more allergens, can be used for treating allergy. An allergen is a substance capable of inducing allergic reactions, i.e., immune reactions mediated by IgE, in a subject (e.g., a human) who has repeatedly exposed to it. It can be any naturally occurring proteins, including pollen allergens (tree-, herb, weed-, and grass pollen allergens), insect allergens (inhalant, saliva and venom allergens, such as mite allergens, cockroach and midges allergens, and hymenoptera venom allergens), animal hair and dandruff allergens (from e.g. dog, cat, horse, rat, mouse etc.), and food allergens. Important pollen allergens from trees, grasses and herbs are such originating from the taxonomic orders of Fagales, Oleales, Pinales and platanaceae including birch (*Betula*), alder (*Alnus*), hazel (*Corylus*), hornbeam (*Carpinus*) and olive (*Olea*), cedar (*Cryptomeria* and *Juniperus*), Plane tree (*Platanus*), the order of Poales including grasses of the genera *Lolium*, *Phleum*, *Poa*, *Cynodon*, *Dactylis*, *Holcus*, *Phalaris*, *Secale*, and *Sorghum*, the orders of Asterales and Urticales including herbs of the genera *Ambrosia*, *Artemisia*, and *Parietaria*. Other important inhalation allergens are those from house dust mites of the genus *Dermatophagoides* and *Euroglyphus*, storage mite e.g. *Lepidoglyphus*, *Glycyphagus* and *Tyrophagus*, those from cockroaches, midges and fleas e.g. *Blattella*, *Periplaneta*, *Chironomus* and *Ctenocephalides*, and those from mammals such as cat, dog and horse, venom allergens including such originating from stinging or biting insects such as those from the taxonomic order of Hymenoptera including bees (superfamily Apidae), wasps (superfamily Vespidae), and ants (superfamily Formicoidae). Important inhalation allergens from fungi are i.a. such originating from the genera *Alternaria* and *Cladosporium*.

Examples of allergen for use in the method include, but are not limited to, house dust mite allergens, grass pollens, tree pollens, and food allergens.

The allergen used can be a purified protein prepared by a conventional method. Alternative, it can be an extract prepared from a natural source of a desired allergen. Typically, an allergen extract contains one or more isoforms of the allergen.

[0019] Either the detoxified LT mutant or a combination of the LT mutant and one or more allergens can be formulated in a pharmaceutical composition (e.g., a vaccine), which can further contain a pharmaceutically acceptable carrier such as phosphate buffered saline or a bicarbonate solution. The carrier must be "acceptable" in the sense that it is compatible with the active ingredient of the composition, and preferably, capable of stabilizing the active ingredient and not deleterious to the subject to be treated. The carrier is selected on the basis of the mode and route of administration, and standard pharmaceutical practice. Suitable pharmaceutical carriers and diluents, as well as pharmaceutical necessities for their use, are described in Remington's Pharmaceutical Sciences.

[0020] Methods for preparing vaccines are generally well known in the art, as exemplified by U.S. Patents. 4,601,903; 4,599,231; 4,599,230; and 4,596,792. Vaccines may be prepared as injectables, as liquid solutions or emulsions. The LT mutant described herein or its combination with an allergen may be mixed with physiologically acceptable excipients, which may include, water, saline, dextrose, glycerol, ethanol, and combinations thereof.

The vaccine may further contain minor amounts of auxiliary substances such as wetting or emulsifying agents, or pH buffering agents to enhance the effectiveness of the vaccines. Vaccines may be administered parenterally (e.g., subcutaneous or intramuscular injection, or transcutaneous delivery) or mucosally (e.g., intranasally, sublingually, or orally). Alternatively, other modes of administration including suppositories, oral, or topical formulations may be desirable. For suppositories, binders and carriers may include, for example, polyalkylene glycols or triglycerides. Oral formulations may include normally employed excipients such as, for example, pharmaceutical grades of saccharine, cellulose, magnesium carbonate and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders.

[0021] The vaccine is administered in a manner compatible with the dosage formulation, and in an amount that is therapeutically effective, protective, and immunogenic. Particularly, the amount of the detoxified LT mutant in the vaccine must be sufficient for reducing Th2-type immune responses. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual's immune system to synthesize antibodies, and if needed, to produce a cell-mediated immune response. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of micrograms of the polypeptide described herein. Suitable regimes for initial administration and booster doses are also variable, but may include an initial administration followed by subsequent administrations. The dosage of the vaccine may also depend on the route of administration and varies according to the size of the host.

[0022] Without further elaboration, it is believed that one skilled in the art can, based on the above description, utilize the present invention to its fullest extent. The following specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

EXAMPLE 1: Suppression of Th2-Type Immune Responses in Asthma Mice by LTS61K Alone or in Combination with Der p Allergen

[0023] Female BALB/c mice (6-8 weeks) were used in this study. These mice were under specific-pathogen free and standard food/water conditions. The mice were treated following the preventive or therapeutic protocol shown in Fig. 1.

Preventive protocol

[0024] Mice were randomly assigned into four groups, each treated with (i) saline (10 μ l per mice), (ii) *Dermatophagoides pteronyssinus* (Der p from Allergon, 20 μ g per mice), (iii) LT(S61K) (10 μ g per mice), or (iv) LT(S61K) (10 μ g) mixed with Der p (20 μ g), per mouse on Day 0, Day 7, and Day 14. Two weeks later, the mice were sensitized with Der p twice (on Day 28 and Day 35) and then subjected to airway challenge on Day 42. They were sacrificed three days later.

[0025] The mice thus treated were examined to determine their AHR levels. More specifically, Penh values, representing dynamic airway resistance, were measured in those mice treated with various concentrations of acetyl- β -methylcholine chloride (methacholine) (Sigma-Aldrich) by Unrestrained Whole Body Plethysmography (Buxco) before sacrifice. As shown in Fig. 2, the mice treated with either LTS61K or the combination of LTS61K and Der p exhibited lower Penh values than those treated with saline and Der p. This result indicates that LTS61K, either alone or in combination with allergen Der p, reduced AHR in asthma mice.

[0026] Bronchoalveolar lavage (BAL) fluids were collected from the mice. The total cell numbers in the BAL fluids were counted after trypan blue staining. The results thus obtained indicate that less cells were present in the BAL fluids from the mice treated with LTS61K and LTS61K/Der p than in the BAL fluids from the mice treated with saline and Der p.

[0027] The levels of certain cytokines, e.g., IL-2, IL-5, TNF- α , eotasin, and TARC, in the BAL fluids were determined

by sandwich ELISA, using analysis kits from e-Bioscience and R&D DuoSet. No difference in Th1-type cytokines, such as IL-2, was detected between the mice treated with LTS61K or LTS61K/Der p and the mice treated with saline or Der p. On the other hand, the levels of Th2-type cytokines, i.e., IL-5, eotaxin, and TARC, were lower in LTS61K- or LTS61K/Der p-treated mice than in saline or Der p-treated mice.

[0028] Further, splenocytes were isolated from the treated mice and cultured in 24-wells plates at a density of 1×10^6 cells/well in cRPMI supplemented with 10%(v/v) FBS, 1mM sodium pyruvate, 50mM 2-mecaptoethanol, 2mM L-glutamine, 1mM non-essential amino acid, 100 U/ml penicillin and 100 μ g/ml streptomycin). The splenocytes were then challenged with 10 μ g Der p for 2 to 3 days and the Supernatants were then collected. The levels of IFN- γ , IL-4, and IL-10 in the supernatants were determined using sandwich ELISA kits provided by R&D DuoSet. In addition, 1×10^5 splenocytes were seeded in a well of a 96-wells plate in the presence of 1 μ g Der p and 0.1 μ g or 1 μ g PHA-L (Sigma-Aldrich). Cell proliferation was examined using a CCK-8 kit (Dojindo) 48 or 72 hour later. While no difference in splenocyte proliferation was observed among the four groups of mice, a lower level of both Th1 and Th2 cytokines was observed in the mice treated with LT(S61K) or LT(S61K)/Der p as relative to that in the mice treated with saline.

[0029] Taken together, the results discussed above show that LTS61K, either alone or in combination with Der p, suppressed TARC and Th2-type immune response when administered to asthma mice following the preventive protocol.

Therapeutic protocol.

[0030] Mice were first sensitized with Der p, which was mixed with incomplete Freund's adjuvant (IFA, Sigma-Aldrich) in equal volume, as follows. Each mouse was injected intra-dermally with 40 μ g Der p on day 0 and day 7 and injected intra-tracheally with 50 μ g Der p under light anesthesia by Zoletil 50 plus Rompun on day 14. The sensitized mice were randomly assigned into four groups, each administered intranasally with (i) saline (10 μ l per mice), (ii) Der p (20 μ g per mice), (iii) LT(S61K) (10 μ g per mice), and (iv) LT(S61K) (10 μ g) mixed with Der p (20 μ g), per mouse under light anesthesia 3 times on Days 16, 18, and 20. At day 21, the mice were injected intra-tracheally with 50 μ g Der p and sacrificed 3 days later (Day 24).

[0031] AHR and the total cells infiltrated into the BALFs were examined as described above. A lower level of airway resistant was observed in the group (ii) and group (iv) mice, as compared with the group (i) and group (iii) mice. Further, the cell numbers in the BALFs from the group (iii) and group (iv) mice were lower than those in the BALFs from the group (i) and group (ii) mice.

[0032] The levels of various cytokines in the BALFs were determined by sandwich ELISA as described above. The cytokines levels, particularly Th2-type cytokines IL-5, TARC, and eotaxin, were significantly lower in the BALFs from the group (iii) and group (iv) mice, treated with LTS61K and its combination with Der p, respectively, than in the BALFs from the group (i) and group (ii) mice, treated with saline and Der p, respectively. See Fig. 3.

[0033] Splenocytes were isolated from the treated mice; their proliferation and cytokine production were determined as described above. No significant difference in splenocyte proliferation was observed in all of the four groups of mice. On the other hand, the levels of IFN- γ , IL-4, and IL-10 were significantly lower in the group (iii) and group (iv) mice, as relative to those in the group (i) and group (ii) mice.

[0034] In sum, the results discussed above show that LTS61K, either alone or in combination with Der p, suppressed TARC and Th2-type immune response when administered to asthma mice following the therapeutic protocol shown in Fig. 1.

EXAMPLE 2: Local and Systemic Induction of Allergen-Specific IgA in Asthma Mice by Intranasal Treatment of LTS61K Alone or in Combination with Der p

[0035] Allergen-specific IgA has been reported to play a protective role in allergic asthma. The levels of Der p-specific IgA were examined in both the mice subjected to the preventive protocol and the mice subjected to the therapeutic protocol (see Fig. 1) as follows. A multi-well plate was coated with Der p (10 μ g/well) in a coating buffer (15mM Na₂CO₃ and 35mM NaHCO₃, p=9.6) at 4 °C overnight and then wash by PBST (0.05% Tween 20 in PBS) and blocked by 0.5% BSA (Sigma-Aldrich). Serum or BAL fluid samples from the mice were diluted and incubated in the coated plate at 4 °C overnight. HRP-conjugated goat anti-mouse IgA antibodies (1:10000, Novus Biologicals) were then added to the plate. After the plate was washed by PBST, tetramethylbenzidine (TMB, Clinical Science Products) was added therein for signal development. As shown in Figs. 4 and 5, the combination of LTS61K and Der p induced anti-Der p IgA production in both blood and BALFs in mice treated following either the preventive or therapeutic protocol.

Claims

1. A detoxified *E. coli* heat-labile enterotoxin (LT) for use in the treatment of airway allergy in a subject via intranasal

administration of an amount effective for inducing an anti-allergic response in the subject, wherein the detoxified LT is LTS61K, which contains a mutated subunit A that contains a serine to lysine mutation at the position corresponding to position 61 in SEQ ID NO:1 and wherein the anti-allergic response is induced by LTS61K.

2. The detoxified LT for use of claim 1, which is formulated in a pharmaceutical composition that is free of allergens.
3. The detoxified LT for use of claim 1, which is formulated in a pharmaceutical composition that contains an allergen.
4. The detoxified LT for use of claim 1, 2, or 3, wherein the subject suffers from or is at risk for asthma.

Patentansprüche

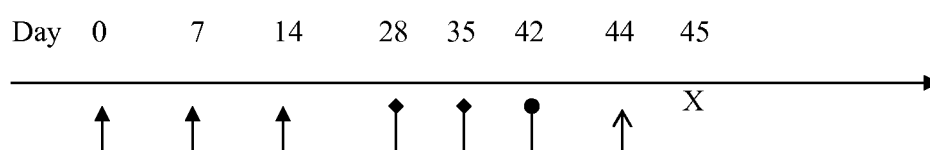
1. Entgiftetes wärmelabiles E.-coli-Enterotoxin (LT) zur Verwendung zur Behandlung von Atemwegsallergien bei einem Individuum durch intranasale Verabreichung einer wirksamen Menge zur Induktion einer antiallergischen Reaktion in dem Individuum, wobei das entgiftete LT LTS61 K ist, das eine mutierte Untereinheit A enthält, die eine Serin-zu-Lysin-Mutation an jener Position enthält, die Position 61 in Seq.-ID Nr. 1 entspricht, und wobei die antiallergische Reaktion durch LTS61 K induziert wird.
2. Entgiftetes LT zur Verwendung nach Anspruch 1, das als pharmazeutische Zusammensetzung formuliert ist, die frei von Allergenen ist.
3. Entgiftetes LT zur Verwendung nach Anspruch 1, das als pharmazeutische Zusammensetzung formuliert ist, die ein Allergen enthält.
4. Entgiftetes LT zur Verwendung nach Anspruch 1, 2 oder 3, wobei das Individuum an Asthma leidet oder gefährdet ist, an Asthma zu erkranken.

Revendications

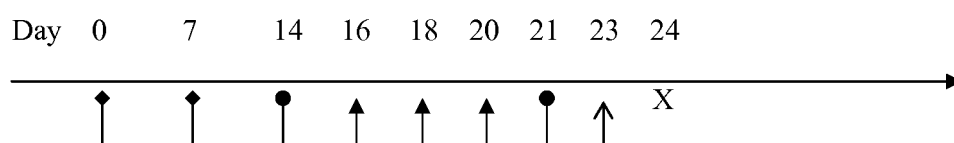
1. Entérotoxine thermolabile (LT) d'*E. coli* détoxifiée destinée à être utilisée dans le traitement de l'allergie des voies respiratoires chez un sujet par administration intranasale d'une quantité efficace pour induire une réponse anti-allergique chez le sujet, dans laquelle la LT détoxifiée est LTS61K, qui contient une sous-unité mutée A qui contient une mutation sérine à lysine à la position correspondant à la position 61 dans SEQ ID NO:1 et dans laquelle la réponse anti-allergique est induite par LTS61K.
2. LT détoxifiée destinée à être utilisée selon la revendication 1, qui est formulée dans une composition pharmaceutique sans allergène.
3. LT détoxifiée destinée à être utilisée selon la revendication 1, qui est formulée dans une composition pharmaceutique qui contient un allergène.
4. LT détoxifiée destinée à être utilisée selon la revendication 1, 2 ou 3, dans laquelle le sujet souffre ou est exposé à un risque d'asthme.

Fig. 1

Preventive protocol:



Therapeutic protocol:



↑ : intranasal treatment with (a) normal saline (10 μ l); (b) Der p (20 μ g); (c) LT(S61K) (10 μ g); or (d) LT(S61K)/Der p (10 μ g/20 μ g)

◆ : sensitization and boostby intradermal injection of 40 μ g Der p/IFA

● : intratracheal airway challenge with 50 μ g Der p

↑ : analyzing airway hyper-responsiveness

x : sacrificed

Fig. 2

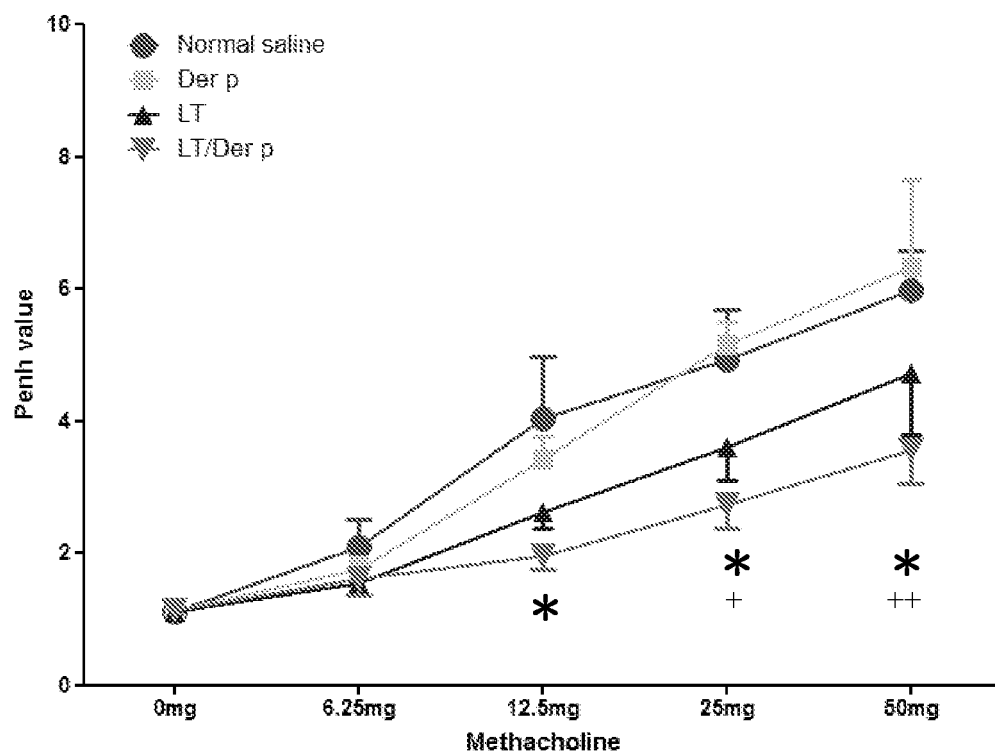
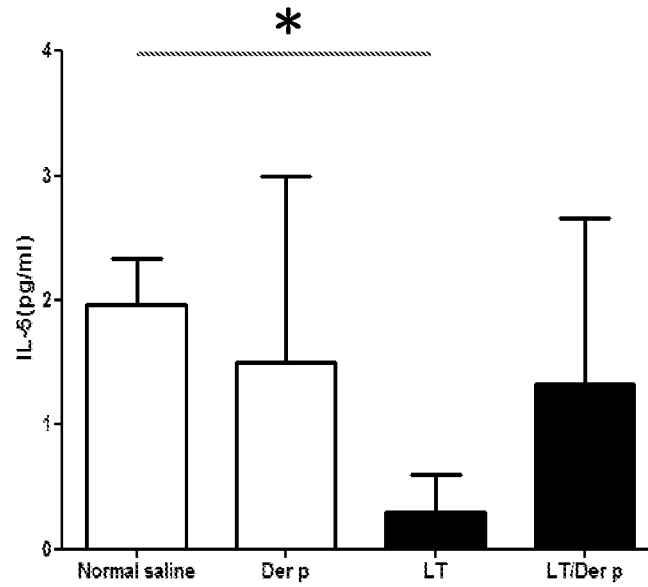


Fig. 3

A. IL-5



B. TNF- α

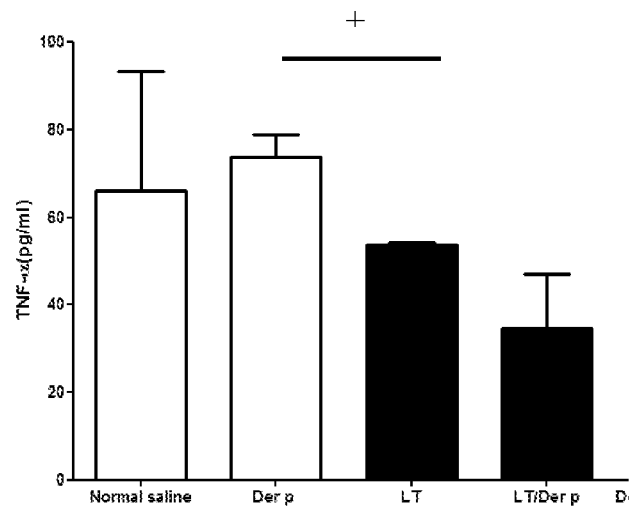
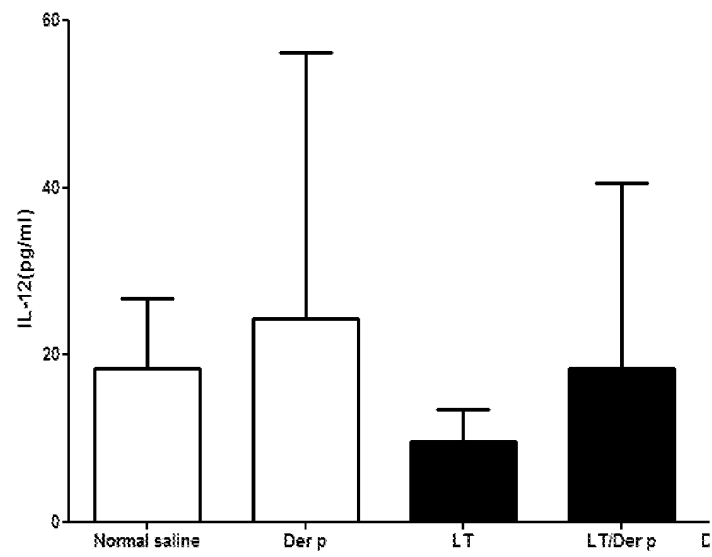
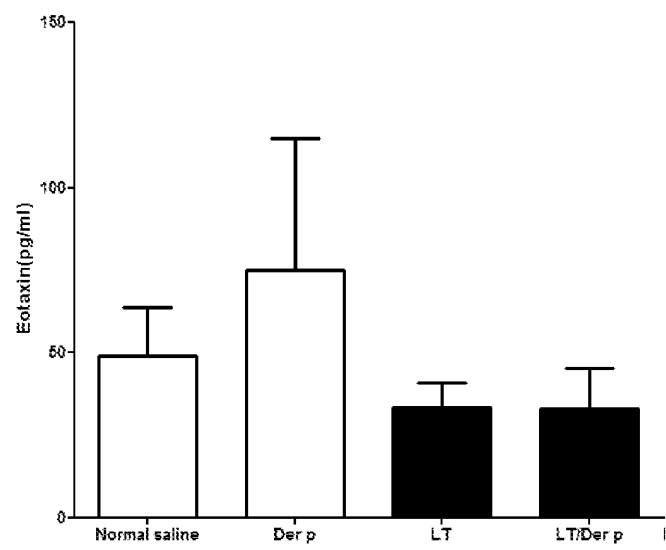


Fig. 3 (Cont'd)

C. IL-12



D. Eotaxin



E. TARC

*

*

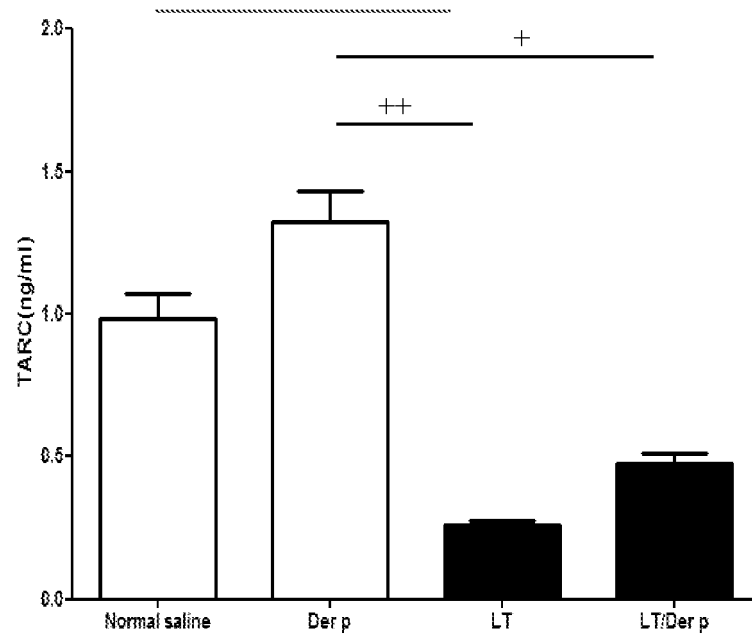


Fig. 4

(a) Serum

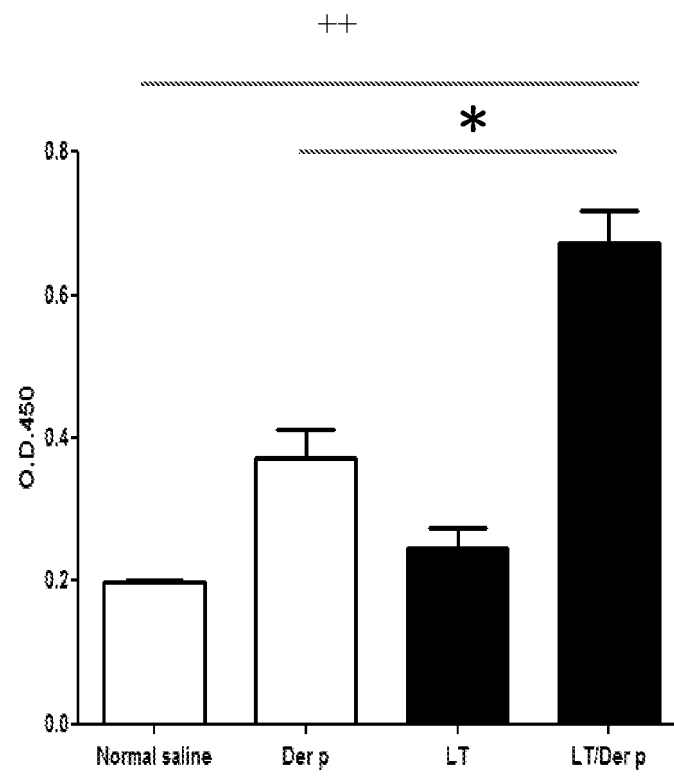


Fig. 4(Cont'd)

(b) BAL fluid

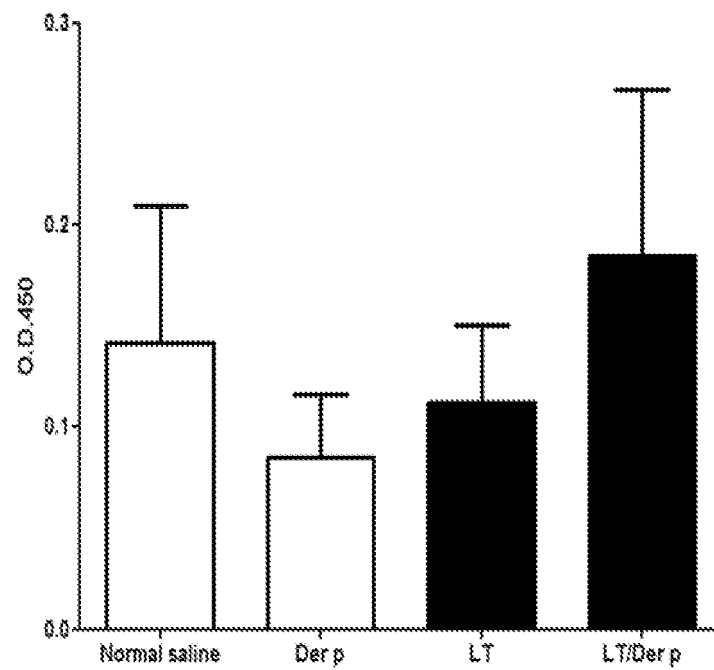


Fig. 5

(a) Serum

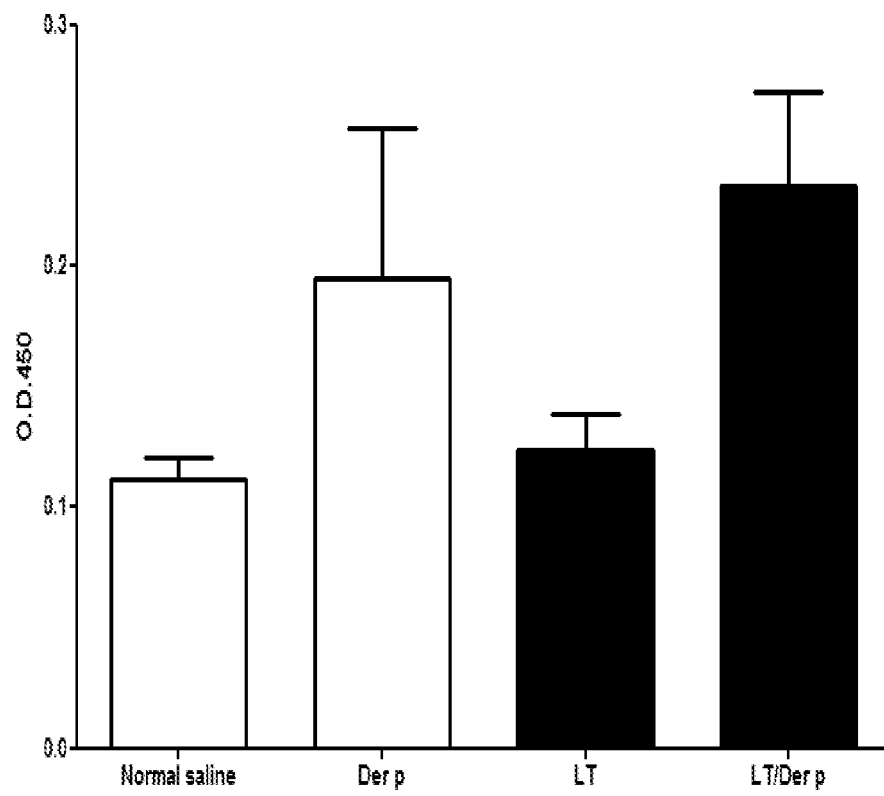
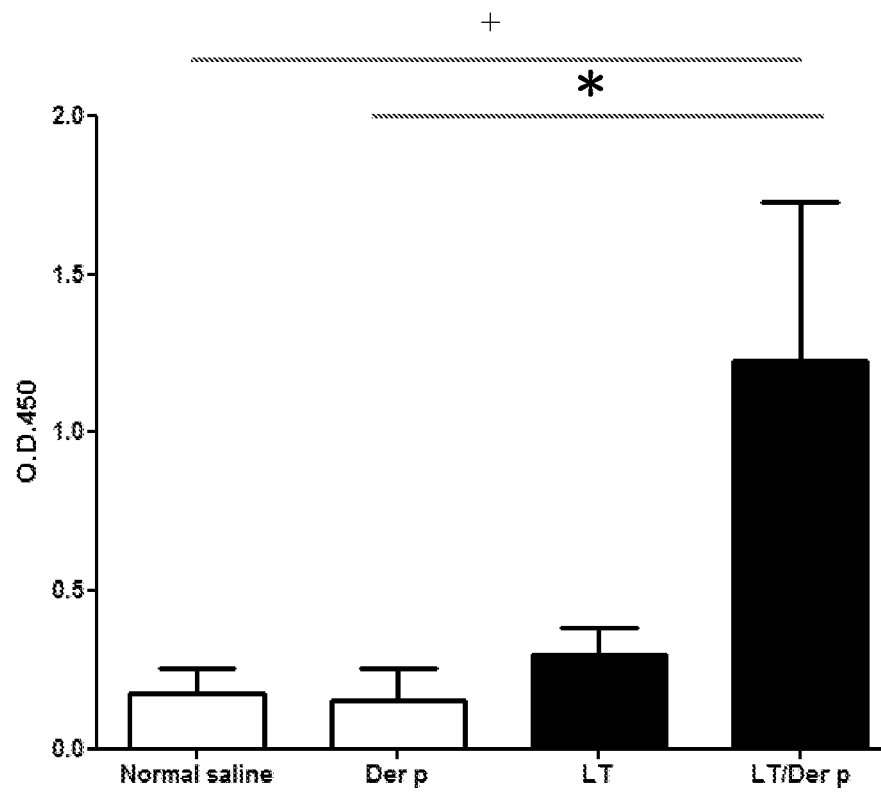


Fig. 5 (Cont'd)

(b) BAL fluid



REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- JP 2003012556 A [0002]
- US 6149919 A [0015]
- US 20081012078 A [0015] [0017]
- US 20010044416 A [0016]
- WO 9947164 A [0016]
- US 6818222 B [0016]
- US 4601903 A [0020]
- US 4599231 A [0020]
- US 4599230 A [0020]
- US 4596792 A [0020]

Non-patent literature cited in the description

- **DAVID et al.** *Infection and Immunity*, 1975, vol. 11, 334-336 [0011]
- **CHENG et al.** *Vaccine*, 1999, vol. 18, 38-49 [0011]
- **GRANT, C.C. et al.** *Infection and immunity*, 1994, vol. 62, 4270-4278 [0011]
- **CHENG, E. et al.** *Vaccine*, 2000, vol. 18, 38-49 [0011]
- **PARK, E. J. et al.** *Experimental and molecular medicine*, 1999, vol. 31, 101-107 [0011]
- **CIEPLAK et al.** *J. Biol. Chem.*, 1995, vol. 270 (51), 30545-30550 [0015]
- **CHENG et al.** *Vaccine*, 2000, vol. 18, 38-49 [0015]
- **NAWAR et al.** *Infection and Immunity*, 2005, vol. 73 (3), 1330-1342 [0016]
- **KOMASE et al.** *Vaccine*, 1993, vol. 16 (2), 248-254 [0016]
- **MAGAGNOLI et al.** *Infection and Immunity*, 1996, vol. 64 (12), 5434-5438 [0016]